WWOX gene restoration prevents lung cancer growth *in vitro* and *in vivo*

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The *WWOX* (WW domain containing oxidoreductase) gene at the common fragile site, FRA16D, is altered in many types of cancer, including lung cancer. We have examined the tumor suppressor function of *WWOX* in preclinical lung cancer models. The *WWOX* gene was expressed in lung cancer cell lines through recombinant adenovirus (Ad) infection (Ad-*WWOX*), and through a drug [ponasterone A, (ponA)]-inducible system. After *WWOX* restoration *in vitro*, endogenous Wwox protein-negative cell lines (A549, H460, and H1299) underwent apoptosis through activation of the intrinsic apoptotic caspase cascade in A549 and H460 cells. Ectopic expression of Wwox caused dramatic suppression of tumorigenicity of A549, H460, and H1299 cells in nude mice after Ad-*WWOX* infection and after ponA induction of Wwox expression in H1299 lung cancer cells. Tumorigenicity and *in vitro* growth of U2020 (Wwox-positive) lung cancer cells was unaffected by Wwox overexpression. This study confirms that *WWOX* is a tumor suppressor gene and is highly effective in preventing growth of lung cancer xenografts, whether introduced through viral infection or by induction of a silent *WWOX* transgene.

adenovirus | inducible expression | viral gene transduction

Lung cancer is the leading cause of cancer mortality in the United States (1), with an incidence of ≈170,000 new cases per year in the United States (1), and mortality is very high. Nonsmall cell lung cancer (NSCLC) accounts for ≈80% of lung cancers. Surgery remains the main therapy for NSCLC, but a large fraction of patients cannot undergo curative resection. Despite new drugs and therapeutic regimens, the prognosis for lung cancer patients has not significantly changed in the last 10 years. Recombinant virus gene therapy has been investigated in lung cancer patients; adenovirus (Ad) and retrovirus encoding wild-type p53 have been injected intratumorally in lung cancer clinical trials (2–6). Recombinant Ad injection in lung cancer phase I studies (7) has demonstrated safety and feasibility, and phase I/II clinical trials are currently recruiting patients to evaluate toxicity and efficacy of gene therapy with recombinant Ads.**

Lung cancer is associated with early loss of expression of the *FHIT* (fragile histidine triad) gene (8) at fragile site FRA3B (9). Fragile regions are particularly susceptible to damage on exposure to environmental carcinogens, which are etiological factors in lung cancer. Recently, Yendamuri *et al.* (10) have demonstrated that the *WWOX* (WW domain containing oxidoreductase) gene is also altered in a fraction of nonsmall cell lung cancers. *WWOX* is located at fragile site FRA16D (11) and encodes a 414-aa protein with two WW domains and a short-chain dehydrogenase domain. WW domains are protein–protein interaction domains, and Wwox interactors with important signaling roles in normal epithelial cells have been identified. Wwox interacts with p73 and can trigger redistribution of nuclear p73 to the cytoplasm, suppressing its transcriptional activity (12). Wwox also interacts with Ap2-γ transcription factors with roles in cell proliferation (13). Most recently, Wwox has been reported to compete with Yap protein for binding to the intracellular ErbB4 domain, a transcriptional activator (14). Thus, the Wwox pathway includes a number of downstream signaling proteins that may also serve as cancer therapeutic targets.

The *WWOX* gene is altered in many types of cancer, including breast, ovary, prostate, bladder, esophagus, and pancreas (15–19). In nonsmall cell lung cancer, transcripts missing *WWOX* exons were detected in 26% of tumors and in five of eight cell lines (10). *WWOX* allele loss occurred in 37% of tumors, and the promoter is hypermethylated in 62.5% of squamous cell lung carcinomas (10, 19). To investigate tumor suppression in lung cancer, we studied *in vitro* and *in vivo* effects of Wwox protein expression in Wwox-negative (A549, H460, and H1299) and -positive lung cancer cells (U2020) by infection with Ad carrying the *WWOX* gene; H1299 cells were also stably transfected with an inducible Wwox expression vector, which allows induction of near physiologic levels of protein. Wwox restoration effectively induced apoptosis *in vitro* and suppressed lung cancer tumorigenicity in nude mice, with no effect on lung cancer cells that constitutively express the Wwox protein.

Materials and Methods

Cell Culture. Wwox-negative A549, H460, and H1299 and Wwox-positive U2020 lung cancer cell lines from American Type Culture Collection were maintained in RPMI medium 1640 with 10% FBS. HEK-293 CymR cells from Qbiogene (Carlsbad, CA) were cultured in DMEM with 10% FBS. H1299 cells do not express p53, whereas A549 and H460 express wild-type p53 (20).

Recombinant Ads and *in Vitro* Transduction. *WWOX* cDNA from normal human liver RNA (Ambion, Austin, TX) was reverse-transcribed by SuperScript First-Strand Synthesis (Invitrogen). Double-stranded cDNA was prepared by PCR amplification using the following conditions: 95°C for 3 min, 30 cycles at 94°C for 30 sec, 65°C for 60 sec, 72°C for 30 sec, and 72°C for 7 min; *WWOX* forward 5′-GCCAGGTGCCTCCACAGTCAGCC-3′ and *WWOX* reverse 5′-TGTGTGTGCCCATCCGCTCT-GAGCTCCAC-3′ primers were used. The cDNA was cloned into Adenovator-CMV5(CuO)-IRES-*GFP* transfer vector (Qbiogene) (11). This vector allows transgene expression driven by the cumate-inducible CMV5(CuO) promoter. An internal

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Abbreviations: Ad, adenovirus; ponA, ponasterone A; moi, multiplicity of infection; PARP, poly(ADP-ribose) polymerase.

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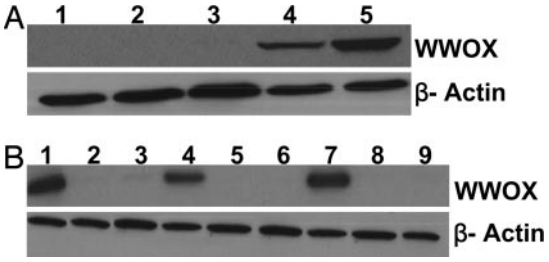

Fig. 1. Expression of Wwox protein. (*A*) Expression of endogenous Wwox is detected in U2020 and MCF7 cells but not in H1299, H460, or A549 cells (50 μg of proteins loaded). Lane 1, H1299; lane 2, H460; lane 3, A549; lane 4, U2020; lane 5, MCF-7. (*B*) Expression of Wwox after infection with Ad-WWOX (25 μg loaded). Lane 1, H1299, Ad-WWOX-infected; lane 2, H1299, Ad-GFP-infected; lane 3, H1299; lane 4, H460, Ad-WWOX-infected; lane 5, H460, Ad-GFP-infected; lane 6, H460; lane 7, A549, Ad-WWOX-infected; lane 8, A549, Ad-GFP-infected; lane 9, A549.

ribosome entry site sequence ensures coexpression of *GFP*. The recombinant plasmid, Ad-*WWOX*, was transfected into modified human fetal kidney HEK-293 CymR cells (Qbiogene) constitutively expressing the CymR protein, which represses the CMV5(CuO) promoter and expression of Wwox during packaging and expansion of the *WWOX* Ad. After 14–21 days, homologous recombination occurred in cells, leading to plaque formation. Plaques were isolated, and viruses were amplified in HEK-293 CymR cells and purified by CsCl gradient centrifugation. Titers were determined by absorbance measurement (number of viral particles per ml) and plaque assay (plaque-forming units/ml), and transgene expression was assessed by immunoblot using Wwox monoclonal antibody (21). Cells were transduced with recombinant Ads at increasing multiplicities of infection (mois) (number of viral particles per cell), and transduction efficiency was determined by visualization of *GFP*-expressing cells.

Inducible *WWOX* Transfectants. The human *WWOX* cDNA was cloned into BamHI and EcoRI sites of the pIND vector. H1299 cells were transfected with 10 μg of pVgRXR vector, which contains the ecdysone nuclear receptor subunits, and clones were selected and tested for ponasterone A (ponA)-inducible expression by transient transfection with a reporter plasmid. Clones showing the highest expression were transfected with 10 μg of the pIND-*WWOX* vector and cultured in zeocin (150 μg/ml) and G418 (1,200 μg/ml). H1299/I clones were selected

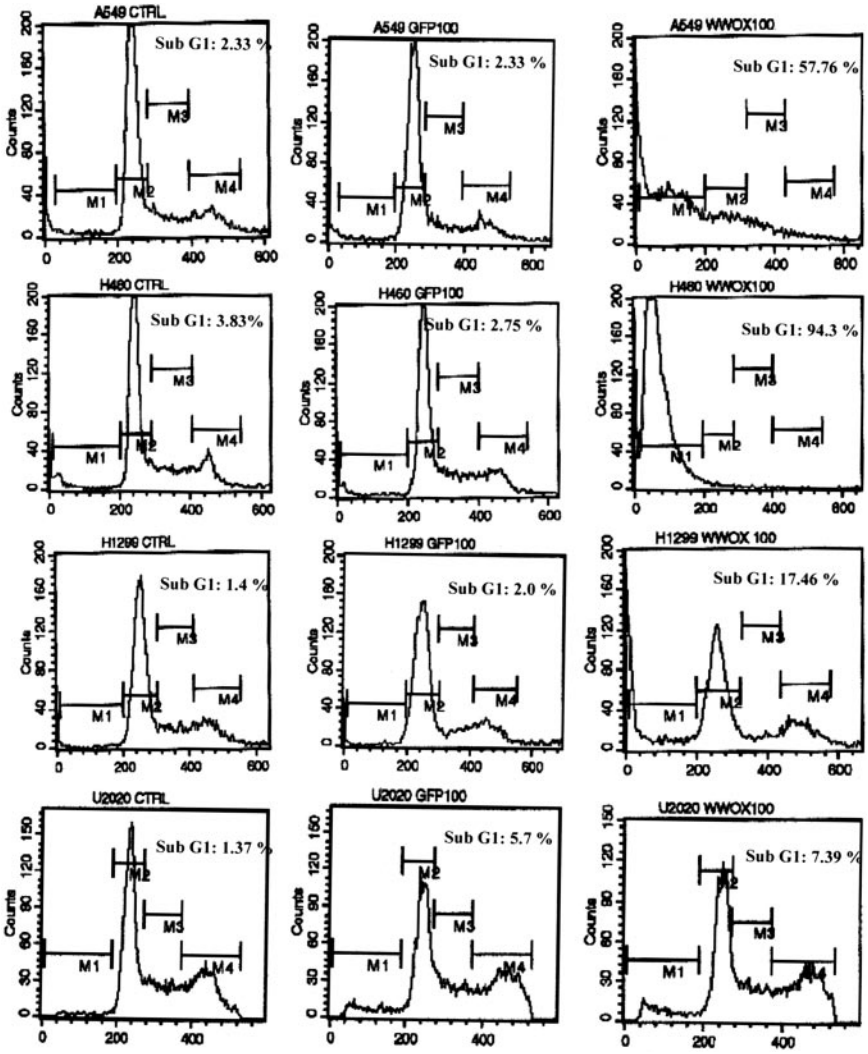

Fig. 2. Flow cytometry analysis of untreated, Ad-GFP-, and Ad-WWOX-infected cells. Wwox-negative A549, H460, and H1299 cells undergo apoptosis 5 days after restoration of Wwox expression by Ad-WWOX infection, but U2020 cells are unaffected. Ad-GFP infection did not induce apoptosis.

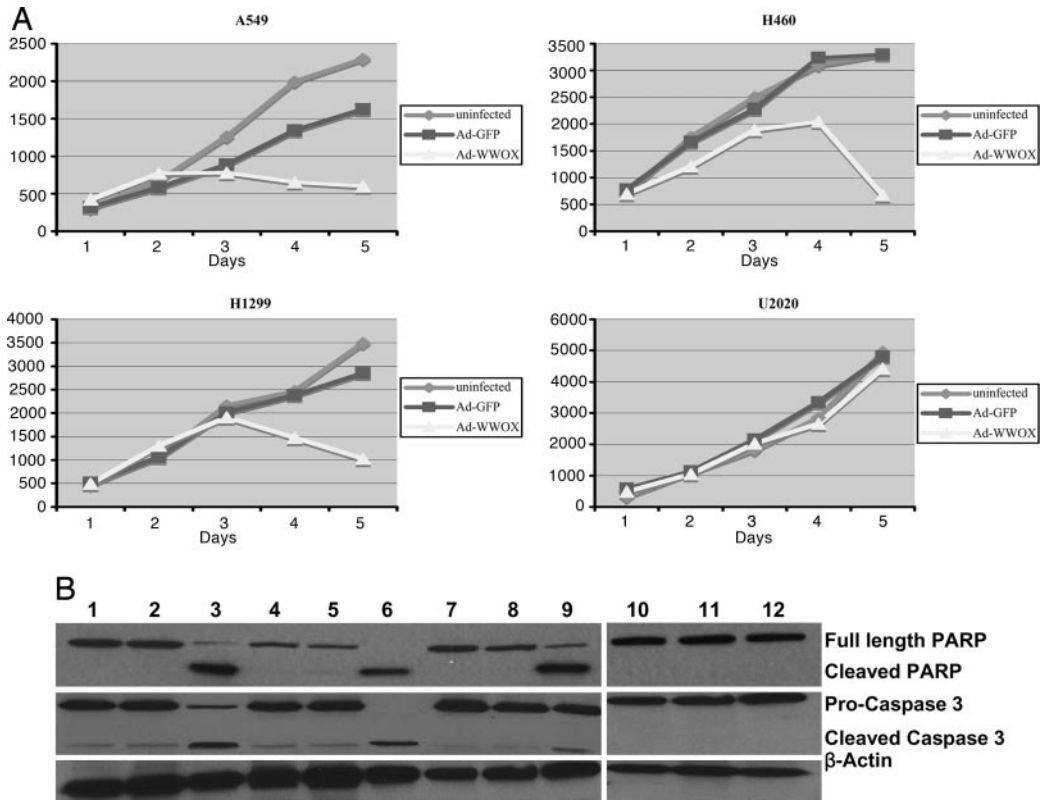

Fig. 3. Effect of Wwox expression on cell growth *in vitro*. (*A*) Growth of uninfected, Wwox-negative A549, H460, and H1299 cells, and cells after infection with Ad-GFP and Ad-WWOX. (*B*) Immunoblot detection of PARP and caspase 3. Lane 1, A549; lane 2, A549/Ad-GFP; lane 3, A549/Ad-WWOX; lane 4, H460; lane 5, H460/Ad-GFP; lane 6, H460/Ad-Wwox; lane 7, H1299; lane 8, H1299/Ad-GFP; lane 9, H1299/Ad-WWOX; lane 10, U2020; lane 11, U2020/Ad-GFP; lane 12, U2020/Ad-WWOX. PARP is cleaved in Wwox-negative cell lines when Wwox is restored through Ad-Wwox infection (lanes 3, 6, and 9). Caspase 3 is cleaved in A549 and H460 (lanes 3 and 6) but not in H1299 cells after Ad-WWOX infection. In U2020 cells, neither PARP nor caspase 3 is cleaved after Ad-WWOX infection (lane 12).

and tested for inducible *WWOX* expression after ponA (5–10 μM) treatment.

Western Blot Analysis. Protein extraction and immunoblot analysis were performed as described in ref. 13. The following primary antisera were used: mouse monoclonal anti-Wwox, 1:500; rabbit polyclonal anti-caspase 3, 1:1,000 (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-caspase 9, 1:200 (Santa Cruz Biotechnology); mouse monoclonal anti-caspase 8 (Cell Signaling Technology), 1:1,000; rabbit polyclonal anti-PARP [poly(ADP-ribose) polymerase], 1:1,000 (Cell Signaling Technology); and rabbit polyclonal anti-β-actin, 1:1,000 (Cell Signaling Technology).

Cell Growth and Cell Cycle Kinetics. Cells (2×10^5) were infected at mois of 10, 25, 50, 75, and 100 and, at 24 hr intervals, were harvested, stained with trypan blue, and counted (ViCell counter, Beckman Coulter). For flow cytometry, cells were harvested 5 days after infection, fixed in cold methanol, RNase-treated, and stained with propidium iodide (50 μg/ml). Cells were analyzed for DNA content by EPICS-XL scan (Beckman Coulter) by using doublet discrimination gating. All analyses were performed in duplicate.

***In Vivo* Studies.** Animal studies were performed according to institutional guidelines. H460, A549, and U2020 cells were infected *in vitro* with Ad-*WWOX* (moi = 100) or Ad-*GFP* or were mock-infected. At 24 hr after infection, 5×10^6 viable cells were injected s.c. into left flanks of 6-week-old female nude mice (Charles River Breeding Laboratories), five mice per infected or control cell line. H1299 cells were infected *in vitro* with Ad-*GFP* or Ad-*WWOX* at a moi of 100. H1299/I cells were treated with 10 μM ponA (H1299/I$^+$ cells) to induce Wwox expression. Tumorigenic controls were uninduced cells (H1299/I$^-$). Induced (H1299/I, 24 hr after ponA treatment) and uninduced (10^7) cells were injected into five nude mice; five mice were also injected with Ad-*WWOX*, Ad-*GFP*, and mock-infected H1299 cells. Tumor diameters were measured every 5 days, and tumors were weighed after necropsy. Tumor volumes were calculated by using the equation V (in mm^3) = $axb^2/2$, where a is the largest diameter and b is the perpendicular diameter.

***Ex Vivo* Studies.** Protein lysates from tumors of H1299, H1299/I$^-$, and H1299/I$^+$ injected mice were evaluated for Wwox expression by immunoblot analysis. Fragments from H1299/I$^+$ tumors were cultured and treated with 10 μM ponA for 2 days to detect expression of inducible Wwox by immunoblot.

Statistical Analysis. Results of *in vitro* and *in vivo* experiments were expressed as mean ± SD. Student's two-sided t test was used to compare values of test and control samples. $P < 0.05$ indicated significant difference.

Results

Wwox Expression in Parental and Ad-*WWOX*-Infected Lung Cancer Cells. Immunoblot analysis of proteins of lung cancer cell lines showed that A549, H460, and H1299 cells did not express endogenous Wwox, whereas Wwox was detected in U2020 cells.

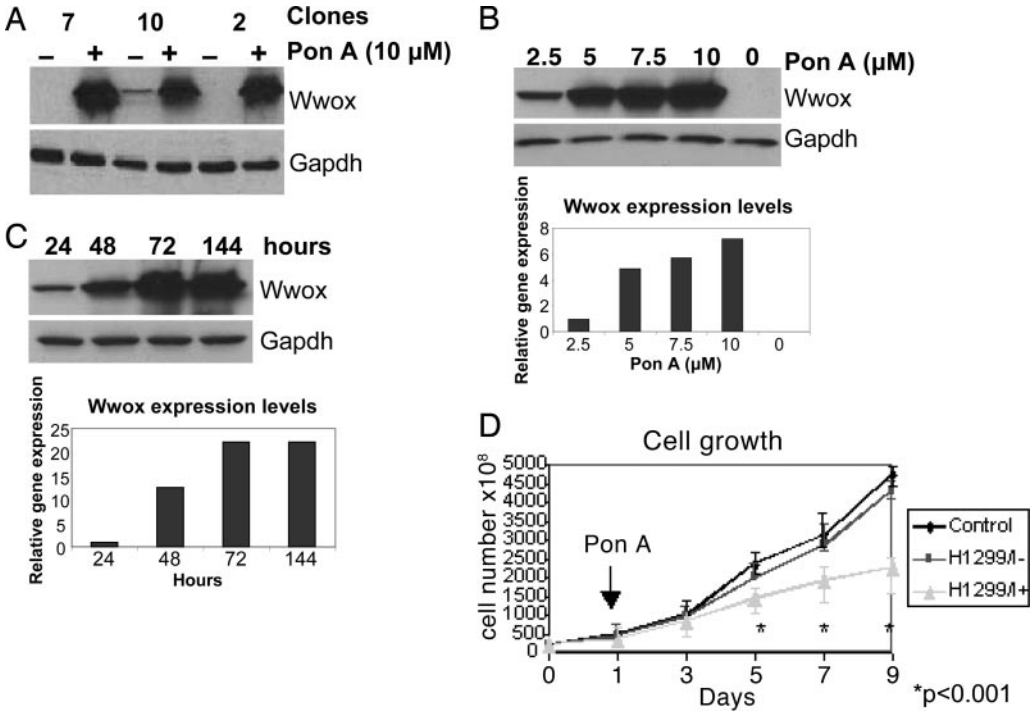

Fig. 4. Inducible expression of Wwox in H1299/I cells. (*A*) Cells were cultured in the presence (+) or absence (−) of 10 μM ponA for 48 hr and tested for Wwox expression. Clones 7 and 2, which expressed the transgene only upon induction with ponA, were used in subsequent experiments. GAPDH expression served as loading control. (*B*) H1299/I clone 7 cells incubated in the absence or presence of increasing concentrations of ponA for 48 hr. Wwox levels increased in a dose-dependent manner and were quantified by densitometry, normalized to GAPDH expression levels. (*C*) Time course of Wwox induction in H1299/I clone 7 cells after treatment with 10 μM ponA. Wwox levels were quantified by densitometry. (*D*) Effect of 10 μM ponA on growth of H1299/I clone 7 cells. On day 1, ponA was added, and maximum Wwox expression was found on day 4. From day 5, the induced cells (H1299/I$^+$) grow significantly more slowly than uninduced cells (H1299/I$^-$) ($P < 0.001$). The experiment was done in triplicate.

Breast cancer MCF-7 cells express abundant endogenous Wwox (18) and served as a positive control (Fig. 1*A*).

Lung cancer cells were infected with Ad-*WWOX* or Ad-*GFP* at a moi of 100; the adenoviral transgene was expressed in nearly 100% of cells of each cell line, as assessed by confocal microscopy of GFP fluorescence (data not shown). Immunoblot analysis 72 hr after infection showed Wwox overexpression in all Ad-*WWOX*-transduced cells (Fig. 1*B*).

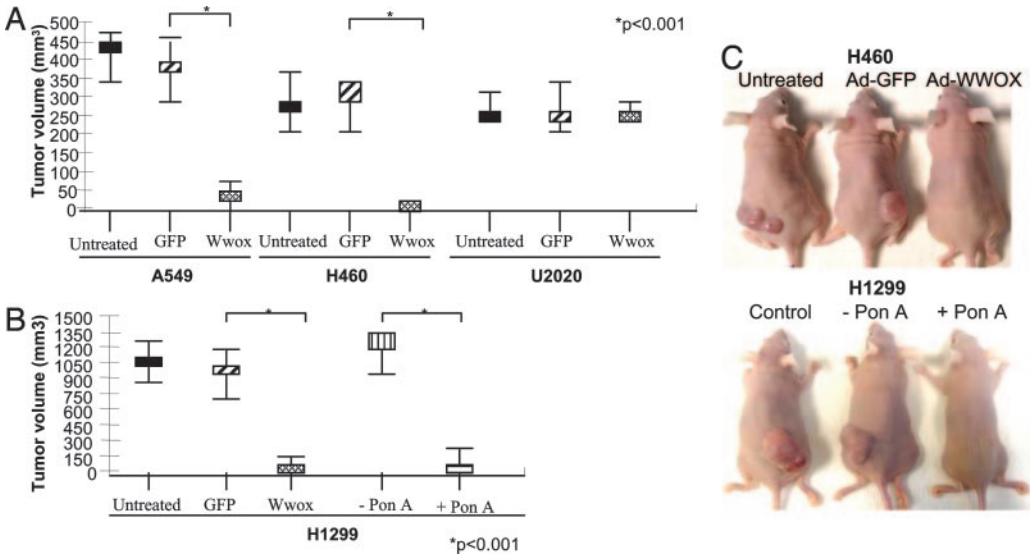

Fig. 5. Effect of Wwox expression on tumorigenicity of lung cancer cells. (*A*) Tumor volume of untreated, Ad-GFP-, and Ad-WWOX-infected A549, H460, and U2020 lung cancer cells. Restoration of Wwox expression in A549 and H460 cells suppressed tumor growth significantly ($P < 0.001$) compared with Ad-GFP infected cells. (*B*) Tumor volume of untreated, Ad-GFP-, and Ad-WWOX-infected H1299 cells and H1299/I$^-$ and H1299/I$^+$ cells. Tumors were suppressed in Ad-WWOX-infected H1299 cells and in H1299/I$^+$ cells. (*C*) Examples of tumor formation by uninfected, Ad-GFP-, and Ad-WWOX-infected A549, H1299/I$^-$, and H1299/I$^+$ cells.

Table 1. Tumor weight (in grams) ± SD in nude mice

	WWOX$^-$			WWOX$^+$
Treatment	A549	H460	H1299*	U2020
Untreated	0.86 ± 0.15	0.64 ± 0.11	1.87 ± 0.33	0.57 ± 0.09
Ad-GFP	0.81 ± 0.16	0.61 ± 0.15	1.66 ± 0.28	0.55 ± 0.05
Ad-WWOX	0.08 ± 0.03	0.03 ± 0.04	0.10 ± 0.26	0.59 ± 0.03

*H1299/I$^-$, 1.98 ± 0.41; H1299/I$^+$, 0.21 ± 0.31.

Cell Cycle Kinetics of Infected Cells. Cell cycle alterations induced by Wwox overexpression were assessed after infection at several mois, with Ad-*WWOX* or Ad-*GFP*. A sub-G_1 population was observed after Ad-*WWOX* infection in A549, H460, and H1299 cells that do not express endogenous Wwox but not in endogenous Wwox-positive U2020 cells. Ad-*GFP* infection did not modify cell cycle profiles. At 96 hr after Ad-*WWOX* infection (moi = 100), 58% of A549, 94% of H460, and 17% of H1299 cells were in the sub-G_1 fraction; 7% of U2020 cells were in the sub-G_1 fraction (Fig. 2). Wwox induction of cell death was moi- and time-dependent (data not shown).

Apoptotic Pathways in Wwox-Reexpressing Cells. A549, H460, H1299, and U2020 lung cancer cell lines were infected with increasing mois, and the fraction of transduced cells was monitored by confocal microscopy and cell cycle kinetics analyses. Significant differences were observed in cell growth for Ad-*WWOX* and Ad-*GFP* infection, at a range of mois, in lung cancer cell lines (A549, H460, and H1299) lacking endogenous Wwox (Fig. 3*A*). U2020 cells were unaffected by exogenous Wwox expression.

To study Wwox-induced apoptotic pathways, expression of downstream apoptotic effectors was assessed *in vitro*. At 96 hr after infection, pro-caspase 3 and full-length PARP-1 levels were reduced in Ad-*WWOX*-infected A549 and H460 cells compared with Ad-*GFP* control cells. In H1299 cells, a decrease of full-length PARP-1 was observed. Cleavage of precursors was not observed in infected U2020 cells (Fig. 3*B*).

Effects of Conditional Wwox Expression in H1299 Cells. H1299/I clone 7 expressed the *WWOX* transgene only on induction with ponA (Fig. 4*A*) and was used in subsequent experiments. Wwox expression increased in a dose-dependent manner after ponA treatment (Fig. 4*B*) from 24 to 72 hr (Fig. 4*C*).

Clone 7 H1299/I$^-$ (uninduced) cells were plated, and, 24 hr later (day 1), Wwox expression was induced by 10 μM ponA. Maximum expression was observed at day 4 and significantly affected cell proliferation by day 5 (Fig. 4*D*), causing reduction in cell numbers and suggesting that Wwox inhibits growth of H1299 cells.

Tumorigenicity of Ad-*WWOX*-Infected Lung Cancer Cell Lines. Nude mice were inoculated with 5×10^6 A549, H460, and U2020 cells infected *in vitro* at a moi of 100 with Ad-*GFP* or Ad-*WWOX* and cultured for 24 hr. Uninfected cells served as tumorigenic controls. At 28 days after injection, tumor growth was completely suppressed in mice inoculated with Ad-*WWOX*-infected H460 cells (Fig. 5*A*). The average tumor weights for controls (Ad-*GFP* and untreated H460 cells) at day 28 were 0.61 ± 0.15 g and 0.64 ± 0.11 g, respectively. At 28 days, two of five mice inoculated with Ad-*WWOX*-infected A549 cells showed no tumors, and average tumor weight was 0.08 ± 0.03 g, significantly lower ($P < 0.001$) than tumors of Ad-*GFP*-infected A549 (0.81 ± 0.16 g) and mock-infected A549 (0.86 ± 0.15 g) cells (Table 1). In mice injected with infected U2020 cells, no tumor growth suppression was observed (Fig. 5*A*).

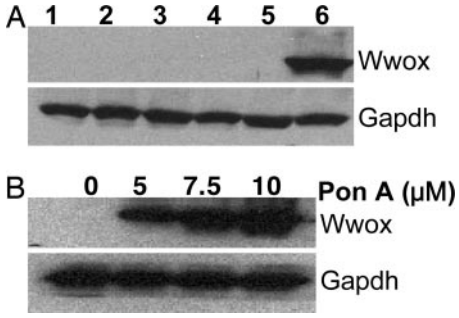

Fig. 6. *Ex vivo* analysis of H1299/I$^-$ and H1299/I$^+$ cells. (*A*) Protein lysates from H1299 (lane 1), uninduced H1299/I$^-$ (lanes 2, 3, and 4), and induced H1299/I$^+$ (lane 5) tumors tested for Wwox expression by immunoblot analysis. Wwox was not expressed in the H1299/I$^-$ or H1299/I$^+$ tumors. (*B*) A portion of the H1299I/$^+$ tumor was plated and cultured, and cells were treated with ponA. Wwox was reexpressed after 48 hr of treatment with 10 μM ponA, indicating the presence of the inducible WWOX plasmid.

Effect of Induced Wwox Expression on Tumorigenicity. We next compared tumorigenicity of H1299 cells infected with Ad-*WWOX* or induced to express Wwox by ponA treatment. Nude mice were inoculated with 1×10^7 cells 24 hr after infection with Ad-*WWOX* or Ad-*GFP*. Five mice were also injected with 1×10^7 uninduced H1299/I (H1299/I$^-$) and 10^7 H1299/I$^+$ cells 24 hr after ponA treatment. At 28 days after injection, three of five and four of five mice inoculated with Ad-*WWOX*-infected H1299 cells and H1299/I$^+$ cells, respectively, displayed no tumors (Fig. 5*B*). Average weight of tumors from Ad-*WWOX*-infected (0.10 ± 0.26 g) and H1299/I$^+$ (0.21 ± 0.31 g) cells was significantly reduced compared with tumors from Ad-*GFP* (1.66 ± 0.28 g), H1299/I$^-$ (1.98 ± 0.41 g), and parental H1299 (1.87 ± 1.33 g) cells (Table 1). Thus, Wwox expression, delivered by viral infection (Ad-*WWOX*) or by induction of expression of an inactive "endogenous" *WWOX* gene (H1299/I$^+$), was effective in suppressing lung cancer cell growth in nude mice.

Wwox Expression in H1299/I$^+$ Explanted Tumors. To assess Wwox expression *ex vivo*, we performed immunoblot analysis of proteins extracted from fragments originating from parental H1299, H1299/I$^-$, and H1299/I$^+$ tumors; Wwox expression was not found in any of the tumors (Fig. 6*A*). Explanted, cultured fragments from H1299/I$^+$ tumors were examined for retention of inducible *WWOX* plasmid by treating with ponA and testing for Wwox expression by immunoblot analysis. The detection of Wwox induction in H1299/I$^+$ explants revealed that the *WWOX* plasmid was present and inducible (Fig. 6*B*), suggesting that the small tumors were derived from inoculated cells that had lost expression of Wwox due to absence of inducer *in vivo*.

Discussion

Innovative therapeutic strategies are urgently needed for lung cancer treatment. Because genes at common fragile sites are frequently inactivated early in the neoplastic process, especially on exposure to environmental carcinogens, we have been interested in the effect of loss of fragile gene expression in development of cancer and therapeutic effects of their restoration (22). A number of studies have suggested that the fragile *WWOX* gene is inactivated in a significant fraction of lung cancers (10, 16), particularly by promoter hypermethylation (16). Hypermethylation is reversible, a strategy with promise for cancer therapy. Thus, we have determined whether restoration of Wwox expression in lung cancer cells lacking expression of endogenous Wwox would reverse malignancy despite numerous cancer-associated genetic alterations that have accumulated in lung cancer cell lines. We have restored Wwox expression in four lung cancer cell

lines by infection with Ad-*WWOX* and observed dramatic loss of tumorigenicity of the lung cancer cells that lacked endogenous Wwox.

Introduction of the *WWOX* gene in the three Wwox-negative cell lines resulted in induction of apoptosis *in vitro*, as shown by the fraction of cells with sub-G_1 DNA content and by suppression of cell growth in culture. The fraction of Ad-*WWOX*-infected H1299 cells with sub-G_1 DNA content was lower than for the other two *WWOX*-negative cell lines, possibly because apoptosis may occur later after restoration of Wwox expression in H1299 cells; another possibility is that expression of p53 in A549 and H460 cells had an additive effect with expression of Wwox protein, although the tumor suppressive effect was similar in the three lung cancer cell lines. The U2020 lung cancer cells expressing endogenous Wwox were not affected by overexpression of Wwox, suggesting that normal Wwox-expressing lung cells would be unaffected by Wwox overexpression after *WWOX* gene therapy. Growth of all three lung cancer cells *in vitro* was adversely affected by overexpression of Wwox after virus infection or ponA induction, as shown by the downturn in cell number after a few days of Wwox overexpression. It will be interesting to examine Wwox binding to known interacting proteins at days 2–5 in these *in vitro* overexpression cultures to define the signal events directly downstream of Wwox expression after *WWOX* infection or induction.

We observed efficient suppression of *in vivo* tumorigenicity of lung cancer cell lines by Ad-*WWOX* transduction in three *WWOX*-negative lung cancer cell lines and by induction of Wwox expression in stably transfected H1299 lung cancer cells. The tumorigenicity of the aggressive H460 cell line was completely suppressed by Ad-*WWOX* treatment at 28 days after injection. A significant reduction in tumor occurrence and size was observed in animals injected with *WWOX*-transduced A549 and H1299 cells. The results suggest that Wwox loss may play an important role in the pathogenesis of lung cancer. It is interesting that both methods of Wwox restoration in H1299 cells appeared to result in more dramatic effects *in vivo* than *in vitro*, possibly because the *in vivo* microenvironment somehow activates the Wwox apoptotic pathway.

This study demonstrates that *WWOX* induces cell growth inhibition and apoptosis in lung cancer cells. In A549 and H460 cell lines, we observed caspase-dependent induction of apoptosis through the intrinsic pathway. In H1299 cells, we observed cleavage of full-length PARP-1, but procaspase 3, 9, and 8 were not cleaved, possibly because apoptosis occurs later in these cells. Wwox and Fhit protein expression is frequently reduced in lung, breast, and bladder cancers in association with promoter hypermethylation (16). Epigenetic alterations can be reversed by specific agents or inhibitors, suggesting such inhibitors as therapeutic agents (23–26). The ponA-inducible expression of Wwox can be considered a model for the effects of *WWOX* reactivation after silencing by epigenetic mechanisms. The extent of loss of tumorigenicity after restoring inducible Wwox expression was comparable to the tumor suppression observed after Ad-*WWOX* expression, both *in vitro* and *in vivo*, suggesting that massive overexpression of Wwox is not necessary to effect tumor suppression. This finding suggests that drugs capable of reactivating the epigenetically silenced *WWOX* gene could be effective in treatment of lung cancer.

In conclusion, restoration of Wwox protein expression in lung cancer cells is followed by induction of apoptosis *in vitro* and suppression of tumorigenicity *in vivo* and suggests that reactivation of the Wwox signal pathway is a potential target for lung cancer prevention and therapy.

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